

Amendments to the Specification:

Please replace the paragraph beginning at page 8, line 7, with the following:

--Fig. 8 is the spectrum of the peptide ladder (SEQ ID NO:1) in the region 87-67 obtained from the mixture 99-67 in Example 2---

Please replace the paragraph beginning at page 8, line 10, with the following:

--Fig. 9 is the spectrum of the mixture 66-33 (SEQ ID NOS:2 and 3) obtained in Example 2.--

Please replace the paragraph beginning at page 8, line 16, with the following:

--Fig. 11 is a spectrum of the reaction mixture (SEQ ID NO:4) obtained in Example 3.--

Please replace the paragraph beginning at page 9, line 1, with the following:

--Figs. 13A and 13B are the mass spectra of the peptide ladders formed from both phosphorylated (12A) (SEQ ID NO:5) (13A) and unphosphorylated (12B) (SEQ ID NO:6) (13B) 16 residue peptides containing a serine residue.--

Please replace the paragraph beginning at page 9, line 7, with the following:

--Fig. 15 shows the spectrum of the mixture (SEQ ID NO:7) obtained in Example 4.--

Please replace the paragraph beginning at page 13, line 21, with the following:

--Thus, for example, if the peaks of the highest mass in Fig. 2 represent a polypeptide, the first five members of which at the amino terminal end may be:

Gly¹-Leu-Val-Phe-Ala⁵-(SEQ ID NO:8),

the next peak of lower mass would represent

Leu²-Val-Phe-Ala⁵-(SEQ ID NO:9)--

Subsequent peaks would represent products with one less amino acid residue. The difference in mass between adjacent members of the series would be indicative of the amino acid residue removed. The difference in molecular mass between the first product on the right and the adjacent product would correspond to a glycine residue. Subsequent peaks show the sequential removal of leucine, valine, phenylalanine and alanine residues thus establishing the sequence of these amino acid residues in the original polypeptide.

Please replace the paragraph beginning at page 28, line 1, with the following:

--A typical example of this aspect of the invention is the analysis of both phosphorylated and unphosphorylated forms of the 16 residue peptide ~~LRRASGLIYNNTLMAR-amide~~
LRRASGLIYNNTLMAR-amide (SEQ ID NO:10) prepared by the method of Schnolzer et al (9) containing a phosphorylated serine residue prepared by enzymatic reaction using 3', 5'-cyclic AMP-dependent kinase. After ten cycles of PITC/PIC chemistry on each form of the peptide using the procedures described above and illustrated in Example 1, the two separate sequence-defining fragment mixtures (peptide ladders) were each read out by laser desorption mass spectrometry. The resulting protein ladder data sets are shown in Figs. 13A and 13B. Again, the mass differences define the identity and order of the amino acids. For the phosphopeptide (Fig. 13A), a mass difference of 166.7 daltons was observed for the fifth amino acid from the N-terminal, compared with the mass difference of 87.0 for the same residue in the unphosphorylated peptide (Fig. 13B). This measured mass difference corresponds to a

phosphorylated serine residue, calculated mass 167.1 daltons. Thus, the protein ladder sequencing method has directly identified and located a Ser(Pi) at position five in the peptide. There was no detectable loss of phosphate from the phosphoserine residue, which has been regarded in the art as the most sensitive and unstable of the phosphorylated amino acids.--

Please replace the paragraph beginning at page 32, line 17, with the following:

--Consideration of the steps involved in the production of a heptapeptide will explain the procedure. If the heptapeptide to be produced is of the structure:

Ala¹-Val-Gly-Leu-Phe-AlaGly⁷ (SEQ ID NO:11),

the first synthetic step is the attachment of Gly to the resin, usually with a spacer molecule between the resin and the Gly. The next step is the attachment of N^α-blocked Ala to the Gly following well known, coupling and deblocking procedures so that the synthesis is controlled. The cycle is repeated to form the heptapeptide on the resin from which it may be isolated by standard methods.--

Please replace the paragraph beginning at page 33, line 5, with the following:

--In accordance with the procedure of this invention, a small sample of polypeptide attached to resin is removed after each cycle. After completion of the synthesis, the seven samples are added together to produce a peptide ladder which contains the following components.

Gly-Resin
Ala-Gly-Resin
Phe-Ala-Gly-Resin
Leu-Phe-Ala-Gly-Resin
Gly-Leu-Phe-Ala-Gly-Resin (SEQ ID NO:12)
Val-Gly-Leu-Phe-Ala-Gly-Resin (SEQ ID NO:13)
Ala-Val-Gly-Leu-Phe-Ala-Gly-Resin (SEQ ID NO:15)--

Please replace the paragraph beginning at page 36, line 15, with the following:

--The procedure will be more readily understood by reference to the preparation of a specific polypeptide such as:

Gly¹-Phe-Ala-Leu-Ile⁵ (SEQ ID NO:16).--

Please replace the paragraph beginning at page 37, line 19, with the following:

--Repetition of these reactions will result in a final resin mixture comprising a peptide ladder which may be represented by:

Fmoc-Ile-Resin
Fmoc-Leu-Ile-Resin
Fmoc-Ala-Leu-Ile-Resin
Fmoc-Phe-Ala-Leu-Ile-Resin (SEQ ID NO:17)
Fmoc-Gly-Phe-Ala-Leu-Ile-Resin (SEQ ID NO:18)
Gly-Phe-Ala-Leu-Ile-Resin (SEQ ID NO:19)--

Please replace the paragraph beginning at page 37, line 19, with the following:

--[Glu¹]Fibrinopeptide B was purchased from Sigma Chemical Co. (St. Louis, Mo.). The reported sequence was: Glu¹-Gly-Val-Asn-Asp⁵-Asn-Glu-Glu-Gly-Phe¹⁰-Phe-Ser-Ala-Arg¹⁴ (SEQ ID NO:20). Matrix assisted laser desorption mass spectrometry gave MW 1570.6 dalton (Calculated: 1570.8 dalton) and showed high purity of the starting peptide. A mixture of PITC plus 5% v/v phenylisocyanate PIC was used in the coupling step. PIC reacts with the NH₂- of a polypeptide chain to yield an N α -phenylcarbamyl-peptide which is stable to the conditions of the Edman degradation. A modification of a standard manual Edman degradation procedure (6) was used. All reactions were carried out in the same 0.5mL polypropylene microfuge tube under a blanket of dry nitrogen. Peptide (200pmoles to 10 nmole) was dissolved in 20uL of pyridine/water (1:1v/v; pH10.1); 20uL of coupling reagent containing PITC:PIC:pyridine:hexafluoroisopropanol (20:1:76:4 v/v) was added to the reaction vial. The coupling reaction was allowed to proceed at 50°C for 3 minutes. The coupling reagents and non-peptide coproducts were extracted by addition of 300uL of heptane:ethyl acetate (10:1v/v), gentle vortexing, followed by centrifugation to separate the phases. The upper phase was aspirated and discarded. This washing procedure was repeated once, followed by washing twice with heptane:ethyl acetate (2:1v/v). The remaining solution containing the peptide products was dried on a vacuum centrifuge. The cleavage step was carried out by addition of 20uL of anhydrous trifluoroacetic acid to the dry residue in the reaction vial and reaction at 50°C for 2 minutes, followed by drying on a vacuum centrifuge. Coupling-wash-cleavage steps were repeated for a predetermined number of cycles. The low MW ATZ/PTH derivatives released at each cycle were not separated/analyzed. Finally, the total product mixture was subjected to an additional treatment with PIC to convert any remaining unblocked peptides to their phenylcarbamyl derivatives. In this final step, the sample was dissolved in 20uL of trimethylamine/water (25%wt/wt) in pyridine (1:1v/v); 20uL of PIC/pyridine/HFIP (1:76:4v/v) was added to the reaction vial. The coupling reaction was carried out at 50°C for 5 min. The reagents were extracted as described above. After the last cycle of ladder generating chemistry, the product mixture was dissolved in 0.1% aqueous trifluoroacetic acid: acetonitrile (2:1, v/v). A

1uL aliquot (250pmol total peptide, assuming no losses) was mixed with 9uL of α -cyano-4-hydroxy-cinnamic acid (5g/L in 0.1% trifluoroacetic acid: acetonitrile, 2:1 v/v), and 1.0uL of this mixture of total peptide products (25pmol) and matrix was applied to the probe tip and dried in a stream of air at room temperature. Mass spectra were acquired in positive ion mode using a laser desorption time-of-flight instrument constructed at The Rockefeller University (7). The spectra resulting from 200 pulses at a wavelength of 355nm, 15 mJ per pulse, were acquired over 80 seconds and added to give a mass spectrum of the protein sequencing ladder shown in Fig. 7. Masses were calculated using matrix peaks of known mass as calibrants.--

Please replace the paragraph beginning at page 47, line 5, with the following:

--Stepwise solid phase synthesis of the 99 amino acid residue polypeptide chain corresponding to the monomer of the HIV-1 protease (SF2 isolate):
PQITLWQRPLVTIRIGGQLKEALLDTGADDTVLEEMNLPGKWKPKMIGGIGGGFIKVRQY
DQIPVEI (Aba) GHKAIGTVLVGPTPVNIIGRNLLTQIG (Aba) TLNF⁹⁹ (SEQ ID NO:21)
[where Aba = α -amino-n-butyric acid] was undertaken.

Highly optimized Boc-chemistry instrument-assisted stepwise assembly of the protected peptide chain was carried out on a resin support, according to the method described by S.B.H. Kent (8). Samples (3-8mg, about 1umole each) were taken after each cycle of amino acid addition. The protected peptide-resin samples were mixed in three batches of consecutive samples: (number corresponds to the amino acid after which sample was taken, i.e. residue number in the target sequence.) 99-67; 66-33; 32-1. The first such mixture contained the peptides:

99-Resin
98-99-Resin
97-98-99-Resin
96-97-98-99-Resin
....(etc.)....
70.... 96-97-98-99-Resin
69-70.... 96-97-98-99-Resin
68-69-70.... 96-97-98-99-Resin
67-68-69-70.... 96-97-98-99-Resin

Similarly for the other two mixtures. The mixed batches of peptide-resin were deprotected and cleaved with HF (1 hours, at 0°C, plus 5% cresol/5%thiocresol). The products were precipitated with diethyl ether, dissolved in acetic acid-water 950/50%, v/v) and then lyophilized.--

Please replace the paragraph beginning at page 49, line 13, with the following:

--Figure 11 shows Figures 9A and 9B show mass spectra of the mixture obtained from mixed samples from residues (66-33) of the synthesis.--

Please replace the paragraph beginning at page 49, line 16, with the following:

--The sequence of the assembled polypeptide chain can be read out in a straightforward fashion from the mass differences between consecutive peaks in the mass spectra of the peptide mixture. This confirmed the sequence of amino acids in the peptide chain actually synthesized. The identity of the amino acids as determined by such mass differences is shown in Table 1 (SEQ ID NO:22).--

Please replace the paragraph beginning at page 52, line 3, with the following:

--Synthesis of the peptide LRRAFGLIGNNPLMAR-amide (SEQ ID NO:23) was performed manually on a 0.2 mmol scale using p-methylbenzhydrylamine resin and 0.8 mmoles amino acid (95 mol% N- α -Boc, 5 mol% N- α -Fmoc) according to the in situ neutralization methods of Schnolzer et al (9). The following side chain protecting groups were used: Boc-Arg, tosyl; Fmoc-Arg, 2,3,6-trimethyl-4-methoxybenzenesulfonyl (Mtr). Fmoc-Arg(Mtr) was used for its greater stability in trifluoroacetic acid (TFA). After completion of the chain assembly, Fmoc groups were removed using 50% piperidine/DMF, followed by Boc group removal in TFA. The peptide fragments were then cleaved from the resin by treatment with HF-10% p-cresol (0°C, 1 hour). The resulting crude peptide products were precipitated and washed with ether, dissolved in 50% acetic acid, diluted with water and lyophilized. The mass spectra of the reaction mixture thus produced is shown in Fig. 11.--

Please replace the paragraph beginning at page 53, line 2, with the following:

--Post-ninhydrin Experiment The machine-assisted assembly of the peptide LRRASGLIYNNPLMAR-amide (SEQ ID NO:24) was performed according to the in situ neutralization methods of Schnolzer and Kent (9) on a 0.25 mmol scale using MBHA resin and 2.2 mmol N- α -Boc amino acids. The following side chain protecting groups were used: Arg, tosyl; Asn, xanthyl; Ser, benzyl(Bz1); Tyr, bromobenzoyloxycarbonyl(BrZ). Resin samples were collected at each step in the synthesis and each sample was individually subjected to the quantitative ninhydrin reaction. These samples were then pooled and the Boc groups removed in neat TFA. Cleavage of the peptide fragments from the resin was performed by treatment with HF-10% p-cresol (0°C, 1 hour). The resulting crude peptide products were precipitated and washed with ether, dissolved in 50% acetic acid, diluted with water and lyophilized. The mass spectrum of the mixture is shown in Fig. 15.--

Appl. No. 10/792,176
Amtd. dated July 21, 2004
Reply to Notice to Comply of May 24, 2004

PATENT

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 11, at the end of the application.

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Amendments to the Drawings:

The attached sheets of drawings includes changes to Fig. 8, Fig. 9a, Fig. 9b and Fig 11. These sheets, which include Fig. 8, Figs. 9a and 9b and Fig. 11 replace the original sheets including Fig. 8, Figs. 9a and 9b and Fig. 11.

Attachment: Replacement Sheets Fig. 8, Figs. 9a, 9b and Fig. 11
Annotated Sheets Showing Changes